A new approach in organ preservation: potential role of new polymers

Thierry Hauet[1,2,3] and Michel Eugene[1,3,4]

[1]Inserm U927, Poitiers, France; [2]INRA, GEPA, Laboratoire de Chirurgie Expérimentale, Domaine du Magneraud, Surgères, France; [3]Faculté de Médecine et de Pharmacie, Université de Poitiers, 6, rue de la Milétrie, Poitiers Cedex, France and [4]Laboratoire de Physiologie et Exploration Fonctionnelle, CHU Poitiers, rue de la Milètrie, Poitiers cedex, France

The storage conditions of the donor kidney may influence the deleterious consequences of ischemia/reperfusion (IR), which remains a major source of complications in clinical practice. Delayed graft function (DGF), seen in 20% to 50% of transplanted cadaver kidneys, is a major risk factor affecting early and long-term graft survival, patient management, and costs of transplantation. Cold preservation plays a key role in this process and is based on hypothermia and high potassium solutions. In this review, the authors focused on the major molecular mechanisms of cold storage (CS) injury at the cellular level, which have been recently evidenced with modern biochemical and cell biologic methods. These newly uncovered aspects of cold preservation injury are often not fully addressed by preservation solutions in current clinical practice. The role of new molecules such as polyethylene glycol (PEG) is presented and their properties are analyzed in the organ preservation context. PEG improves organ function recovery and reduces inflammation and fibrosis development in several models. Because organs shortage is also a real public health problem, organs from non-heart beating donors or marginal donors are now used to expand pool of organs. As a consequence, the development of better organ preservation methods remains a major target and deserves scientific consideration.

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Correspondence: *Thierry Hauet, Inserm U927, Pole UBM, CHU de Poiters, rue de la Miletrie, Poitiers cedex 86019, France. E-mail: t.hauet@chu-poitiers.fr or Michel Eugene, Laboratoire de Physiologie et Exploration Fonctionnelle, CHU, rue de la Miletrie, Poitiers cedex 86019, France. E-mail: m.eugene@chu-poitiers.fr*

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Till date, the procedure of organ transplantation requires the grafted organ to go through phases of cold preservation and warm reperfusion. The resulting ischemia–reperfusion injury (IRI) typically causes early nonfunction or delayed graft function, which plays an important role in the development of chronic graft failure and late graft loss (Figure 1).[1] In addition, the shortage of donors has led many transplant centers to consider accepting older donors with co-morbidities, more prone to delayed graft function. Consequently, the incidence of delayed graft function remains high and the importance of the preservation procedure is now well established.

BASIC PRINCIPLES OF ORGAN PRESERVATION

The basic principles of organ preservation rely on two empirical dogmas: (i) low-temperature preservation and (ii) high-potassium solutions.[2] Low temperature is used to decrease the metabolic rate of a nonperfused organ; however, it can also induce some deleterious effects, and even if it remains the most commonly used strategy, there is still room for the development of preservation procedures at higher temperature, room temperature, or even normothermia, especially when perfusion is associated with preservation.

The high-K^+ dogma relies on the hypothesis that as the function of ionic pumps is impaired during preservation, the intracellular ionic content can be maintained using an extracellular preservation solution with a similar ionic content. This concept is offset by several observations:[1,3] (i) high potassium induces cellular depolarization and accelerates the decrease in the cellular ATP content; (ii) voltage-dependent channels, such as calcium channels, are activated; and (iii) the consecutive calcium influx depends on the potassium concentration of the solution. Most importantly, potassium concentration above 15 mmol/L is a potent stimulus of vasoconstriction that impairs organ perfusion during washout and reperfusion.

Despite the evolution of preservation techniques—at various temperatures and with or without continuous pulsatile perfusion—major factors determining organ viability before transplantation should be taken into account.

One of the most important is tissue edema. Many events contribute to cellular and mitochondrial swelling during

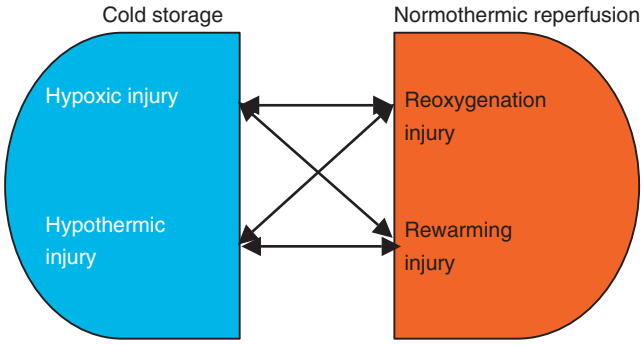

Figure 1 | Several pathophysiological processes involved in organ preservation and reperfusion injury. Preserved and reperfused organ injury is triggered by hypoxia/reoxygenation and by hypothermia/rewarming.

preservation. The initial mechanism was believed to be the inhibition of Na^+/K^+ ATPase followed by intracellular accumulation of sodium, chloride influx, and cell swelling. This mechanism was the corner stone to validate the use of 'intracellular ion content' preservation solutions associated with high concentrations of osmotically active substances.

Suppression of oxidative phosphorylation, ATP depletion, and increase in the cytosolic calcium concentration were widely regarded as consequences of the inhibition of Ca^{2+} ATPase and the reverse mode of function of the Na^+/Ca^{2+} antiporter. Mitochondrial calcium overload follows this increase in cytosolic calcium, and is associated with the opening of the permeability transition pore and mitochondrial swelling.[1,2] This mitochondrial swelling was correlated with proapoptotic events.

The second factor, which impacts organ viability, is oxidative stress.[1,3] Recent reports have shown that the primary mechanism of hypothermic injury was related to free iron release of microsomal origin.[1] Mitochondria are a major cellular source of free radicals and their role was underlined by the increased expression of MnSOD messenger.[1,3] These data emphasize the role of mitochondria in hypothermic and cold-induced injury, which are mediated by reactive oxygen species (ROS), with the cellular labile iron pool playing a decisive role in this injury. The main oxidative stress occurs at the time of reperfusion, when oxygenated blood flow is restored. The reintroduction of oxygen molecules is characterized by the univalent reduction of dioxygen molecules, leading to ROS production and direct injury to cellular structures as well as activation of different cellular signaling pathways.[4] Both ROS and calcium can strongly contribute to the opening of the mitochondrial permeability transition pore.[1,3] Strategies to alleviate ROS-related damage should be aimed at limiting their production during cold preservation and at the time of reoxygenation and rewarming rather than the more difficult approach of neutralizing the already produced ROS.

A third factor is the preservation of cell membrane integrity, and, particularly, that of endothelial cells, which are in direct contact with the cold storage solution during preservation. In addition, it is important to maintain the integrity of the glycocalyx (Gcx),[5] which provides both protection against swelling and limits the visibility of allogenic molecules at the surface of the membrane of the endothelial cell.

NEW PRESERVATION SOLUTIONS: THE ERA OF NEW POLYMERS AND THE PURSUIT OF PERFECTION

Among the key components of preservation solutions, colloids are essential in preventing or minimizing interstitial edema. Hydroxyethyl starch was chosen for the preparation of the UW solution, but was found to cause increased red blood cell aggregation. Furthermore, glutathione added in UW solution for its antioxidant property was shown to be oxidized with time.[1] Since then, other colloids have emerged, notably polyethylene glycol (PEG), which have physical and chemical properties of particular interest in organ preservation.[6]

Immunocamouflage

An alternative to the transplantation tolerance paradigm is the 'immunocamouflage' of foreign cells, tissues, and organs.[7] Immunocamouflage relies on the modification of the cell membrane surface with non-immunogenic molecules, such as PEG molecules, creating a barrier that prevents the recognition of allogenic sites on the cell membrane by circulating cells and antibodies of the recipient. In contrast to other immunomodulatory approaches, immunocamouflage presents a more versatile effect combining interferences with binding, allorecognition, and presentation pathways.[7] To understand this mechanism, we must consider the so-called immunological synapse.[8]

The immunological synapse

T cells are normally activated through direct contact with an antigen-presenting cell. The immunological synapse organizes and segregates functional antigen (LFA-1) and T-cell receptor (TCR) molecules on host lymphocyte membrane from adhesion molecules (ICAM-1) and MHC (major histocompatibility complex) molecules on donor antigen-presenting cells. The interaction between T cells and antigen-presenting cells requires membranes to be in a close proximity: 15 nm at the MHC and TCR level and 45 nm between adhesion molecules.[9] (Figure 2, left panel)

A sustained TCR engagement can be impeded by the small size of the TCR and MHC molecules and, furthermore, engagement of TCR with antigens requires a rearrangement of proteins at the membrane surface, emphasizing the importance of cell membrane fluidity. All these events can be dramatically disturbed if polymers interact at the membrane surface of donor cells, increasing the distance between receptors and ligands, and reducing the lateral mobility of molecules. This is the case with PEG molecules of molecular weight (MW) greater than or equal to 20 kDa, at a sufficient concentration. For PEG 20,000, the adsorption of

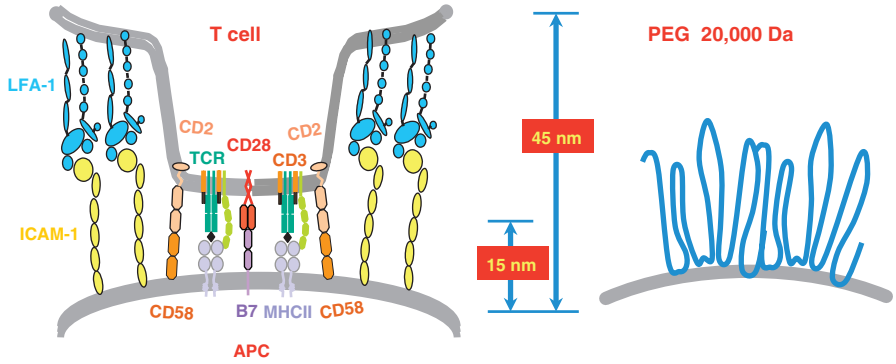

Figure 2 | Immunological synapse and adsorbed PEG molecules of respective sizes. Left: the relative size of the signaling and adhesion molecules involved in the formation of the immunological synapse between a lymphocyte T Cell and an antigen-presenting cell (APC). Right: size at the identical scale of an adsorbed molecule of polyethylene glycol (PEG) of 20,000 Da MW.

the molecule at the membrane surface produces an exclusion space of roughly 15–20 nm in length, which is approximately the size of the immunological synapse.

Nevertheless, PEG molecules adsorbed at the cell membrane surface are eventually cleared, as after kidney transplantation, the urinary excretion of PEG lasts roughly 1 week. However, the long-term effect of a transitory camouflage of cell surface antigens is not fully explained. According to the Danger Signal theory,[10] IRI strongly triggers the recipient immune system (both innate and adaptive). This acute, short-lived situation is known to have deleterious long-term effects. Therefore, it is likely that a transient reduction of antigen presentation (and thus allorecognition) during the acute phase of the inflammatory process (danger signal) would minimize the response of the recipient immune system.

Possible polymers candidates

Numerous hydrophilic polymers can spontaneously bind to cells and tissues, such as dextran (W23), Tween, Brij, polymers with phospholipid polar groups, hyaluronic acid, alginic acid, heparin, albumin, and PEG. However, PEG seems to be the more effective at sterically stabilizing underlying surfaces (Figure 2, right panel).

Polyethylene glycols

The outstanding protection provided by this polymer has been attributed to its molecular properties, such as its low interfacial energy, three-dimensional conformation, hydrophilicity, and high flexibility. PEG 20,000 is a polymer of ethylene oxide with a hydroxyl terminal (Figure 3). The longer chains are also referred to as polyethylene oxide, $HO\text{-}(CH_2CH_2O)_nCH_2\text{-}CH_2\text{-}OH$. The length of the chain determines the MW of the molecule, that is, PEG 20,000 has a MW of 20 kDa.

The possible therapeutic use of PEG is underlined by its chemical properties: PEG is a neutral, water soluble, nontoxic polymer. Its unusual water solubility is the result of a specific water-structuring effect along the backbone of PEG molecules. Thus, with a molecule of PEG 20,000, 8000–10,000 water molecules have reduced mobility and are highly 'structured'. This leads to the creation of a large 'exclusion volume', which prevents the approach of the other molecules. Consequently, PEG is not immunogenic, and behaves as a 'stealth molecule' or a voluminous water cluster. The adsorption or exclusion of PEG molecules at the membrane surface depends on its MW: high MW (>10,000 Da) PEG is adsorbed to the membrane surface and stabilizes it, whereas lower MW PEGs will be repelled from the membrane surface, and the depletion–attraction mechanism will lead to membrane fusion (PEG 8000 or 10,000). There is a critical chain length above which a sufficient number of segments form physical bonds with the surface, and large chains are preferentially adsorbed.

PEG molecules adsorbed to the cell membrane surface can exert a long-range effect impeding the interaction between surface molecules roughly up to 50 nm for a MW of 20 kDa. In a bacterial model (*Pseudomonas aeruginosa*), the adsorption of PEG 15–20 kDa at 10% during 4 h and subsequent washing induced a height deflection of the polymer/bacterial surface of 1500 nm as measured by atomic force microscopy.[11]

Thus, PEG molecules behave like 'stealth molecules' surrounded by a large cluster of structured water with a near 'ice-like' structure. This layer at the cell membrane surface contributes to the reduction of osmotic cell swelling and permeability in numerous models.

PEG and glycocalyx

Receptors and signaling molecules at the cell surface are associated with other membrane-associated proteoglycan, glycosaminoglycans, glycoproteins, and glycolipids to constitute the highly hydrated Gcx. The Gcx, by modulating its density and thickness, is considered a physiological means of regulating cell adhesion. It also contributes to the regulation of cell volume.[5] During ischemia and reperfusion, Gcx integrity can be drastically altered.[5,12] A decrease in Gcx thickness exposes more signaling molecules, such as adhesion molecules, according to their own size, and contributes to the activation of coagulation. The Gcx is severely modified

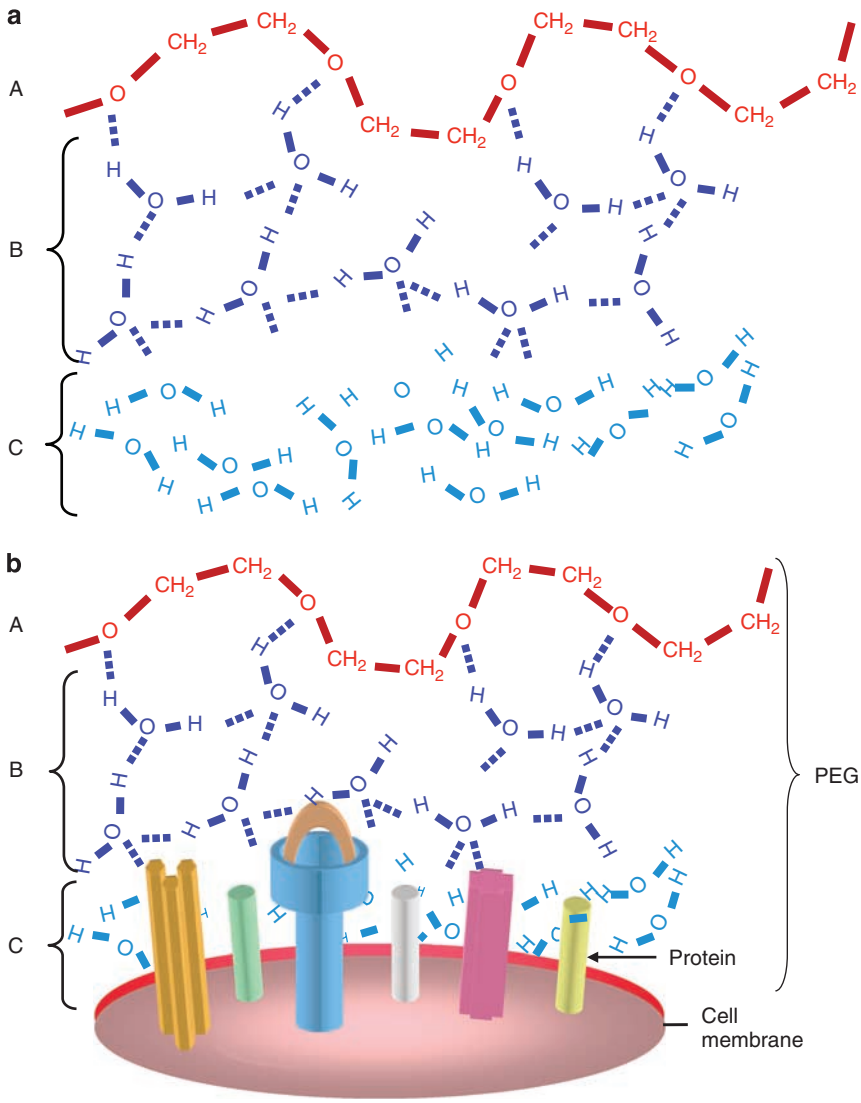

Figure 3 | PEG structure and hydratation layers. (**a**) A, PEG molecule backbone; B, cluster of structured water molecules bonded to PEG molecule with long-lasting hydrogen bonds (dashed lines) (ice-like water); C, Area of dense water molecules with hydrogen bonds of very short duration (liquid water). (**b**) Relation between the PEG structure and the size of proteins at the cell membrane surface.

during IR, when a two-third reduction of its thickness can be observed, likely reducing its protective effect against edema and increasing the visibility of adhesion molecules and other signalization molecules expressed at the membrane surface. PEG molecules could protect or repair the Gcx, restoring its regulatory function,[13] and contribute to immunocamouflage.[14]

PEG and oxidative stress

Polyethylene glycol has demonstrated membrane protective effects in a variety of cells or organs against many different types of insults. Moreover, PEGs are known to decrease ROS elevation and lipid peroxidation in these injury models. However, *in vitro* testing using cell-free systems demonstrated that PEGs do not have the ability to scavenge the superoxide anion DPPH (1,1-diphenyl-2-picrylhydrazyl) or the ability to inhibit xanthine oxidase.[12,13] It is thus likely that PEGs inhibit or reduce oxidative stress mainly through the preservation or restoration of membrane integrity and, thus, could protect against ROS production during IR. Furthermore, the prevention of edema protects against mitochondrial swelling, which itself is a factor contributing to the increase in ROS generation.

Pre-clinical evaluation of PEG

There is evidently a need for pre-clinical *in vivo* models, which are probably more consistent for the evaluation of IRI in transplantation. Many types of cultured cells undergo apoptosis in the absence of specific survival factors, including mechanical factors such as shear stresses.[15] There is a big difference between a flat layer of cells (two-dimensional) and a complex three-dimensional tissue, questioning the relevance of the study of IR and low-temperature effects in two-dimensional cell culture on issues of organ preservation.

Previous evaluation by different laboratories has shown that PEG is a viable strategy toward organ preservation when added in a simplified crystalloid and extracellular solution.

To the best of our knowledge, the first published work concerning the immunosuppressive effect of PEG-containing solutions in humans is by Collins *et al.*[16] They replaced hydroxyethyl starch in a modified UW solution by PEG 20 kDa (50 g/L) to avoid osmotic swelling, and used it as a preservation solution in cardiac transplantation in humans. They report that, unexpectedly, the rate of acute rejection was lower in the PEG group. They suggest a direct effect of PEG solution on the immune process. The same immunoprotection was achieved when they used the PEG 20,000 solution for the preservation of liver, small bowel, and pancreas. Furthermore, the immunosuppressive effect of PEG was dependent on the MW of the molecule. PEG 8000 was ineffective, whereas when using PEG 20,000, the survival of rats after small bowel transplantation was increased, and moreover they did not show signs of acute rejection.[17]

More recently, the potential of PEG in organ preservation has been demonstrated in kidney transplantation. In a model of renal autotransplantation in the pig, the authors demonstrated an absence of side effects in terms of recovery of organ function and by showing a reduction in inflammation and fibrosis development.[6,18] In this model, kidney preservation using PEG 20 kDa combined with extracellular solution resulted in a faster renal recovery than UW solution. In this model, PEG reduced tubular atrophy and fibrosis at 3 months after autotransplantation. Interestingly, additional effects of PEG were a strong reduction of MHC class II expression in epithelial tubule cells, a reduced number of $CD4^+$ T lymphocytes, limited infiltration of macrophages/monocytes, and a lower progression of interstitial fibrosis in the 8- to 12-week post-transplanted kidneys. These processes are an important component of IRI, related to the development of chronic injury, and make the graft more susceptible to rejection.[18] Considering the composition of the solution, these effects can only be attributed to the presence of PEG. Moreover, because this evidently efficient prototype remains a simplified solution, there is room for other additional molecules or biochemically active components, which could be of interest to protect against cold ischemic injury and the coupled effect of reperfusion and rewarming. In addition, PEG was shown to induce an immunoprotection of pancreatic islets transplants in an acute model of rejection.[19] This effect was observed using PEG dissolved freely in the solution, but reactive species of PEG have also been used for the protection of pancreatic islets. Activated PEG allows covalent binding ensuring amine, carboxyl, hydroxyl, thiol, or N-terminal PEGylation at the membrane surface. For example, multiple PEGylation affected neither islet viability nor functionality and, when allotransplanted into diabetic recipients, these islets survived in three of the seven recipients for more than 100 days without any immunosuppressive treatment. In contrast, unmodified islets were completely destroyed within 1 week.[20] These data are encouraging, but it should be mentioned that reactive PEG cannot be used for cold storage, as there are issues with organ perfusion at low pH. Nevertheless, these reactive PEGs are well suited for preservation of islets, isolated large arteries, or heart valves.

Clinical studies are in progress in different multicenter studies. Preliminary results show that PEG is safely usable in human kidney transplantation and provides a good quality of preservation at least equivalent to UW.

CONCLUSION

New concepts regarding organ preservation and limitation of IRI are emerging. The necessity to use low-K^+ solutions to avoid vasoconstriction and high-molecular-weight molecules to limit edema is obvious. Water-soluble polymers, such as high-molecular-weight PEGs, can protect biological surfaces in a nonspecific fashion. A PEG molecule combines two protective actions: (i) it exerts an oncotic pressure that limits the deleterious effects of edema and (ii) it is sufficiently adsorbed at the cell membrane surfaces to stabilize membrane lipids and ensure the immunocamouflage of antigenic sites and may further enhance the immunoprotection of donor cells, tissues, and organs.

Immunocamouflage depends on the size of the exclusion volume around PEG molecules. For nonactivated PEGs, adsorption at the membrane surface is observed preferentially for PEG 20,000. This MW ensures an exclusion layer of about 15–20 nm. The use of activated PEGs allows to fit the PEG size—and thus the exclusion volume—to the size of the antigens of interest. It is evident that the MW of PEG does matter, as PEG 8000 is not effective in ensuring immunoprotection and biological data, illustrating that protection against IRI is obtained using mostly PEG 20,000. This effect can be undoubtedly observed with higher MWs, as the limit is still unknown.

The main advantage of immunocamouflage is that it directly modifies the inherent immunogenicity of the donor tissue itself, using means that are strictly physicochemical in nature and do not rely on the details of activation pathways, leaving the immune system of the recipient fully competent.

DISCLOSURE

All the authors declared no competing interests.

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